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Biocides for the Battlefield—Interim Report

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Table of Contents

List of Figures

List of Tables

Biocides for the Battlefield - Interim Report

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Background

The ability to disinfect surfaces and manufacture self-decontaminating surfaces has been the subject of research within the Chemistry Division at the Naval Research Laboratory. Through several previous work units, a significant synthetic undertaken was made in preparation of a variety of novel highly mobile biocides possessing low surface energies.[1-4] In addition, significant achievement in synthesis of novel classes of biocides were made, with the subsequent incorporation of these molecules into a variety of hydrogel coating systems.[5-7] Further exploration of amphiphilic molecules to achieve surface modification has also been undertaken in low loading conditions.[8-11] This work serves as a solid background onto which the molecules within this report are based.

Surface Self-Concentrating Amphiphilic Quaternary Ammonium Biocides as Coating Additives

The rational design of effective self-decontaminating surfaces has been the goal of a number of research groups,[12-16] and a variety of methods for the surface-modification of polymeric materials have been utilized towards the synthesis of antimicrobial coatings for use in hospitals and in defense of biological weapons.[17] The use of biocidal additives in paints and coatings imparts antimicrobial properties without the need for complex or costly post-manufacturing steps associated with surface-modifying post-treatments.[18] For this purpose the self-concentration of additives at the surface is advantageous not only to preserve the physical properties of the bulk material, but also to increase the antimicrobial activity. Furthermore, a surface-concentrated additive is more efficient than an additive that is evenly distributed throughout the coating, as much of the additive in the latter case will be ineffective within the bulk material. One challenge in the development of such an additive is to impart the ability to surface-concentrate without leaching from the surface, as this invokes environmental concerns.[19] Moreover, while the release of biocidal agents can be an effective method of surface self-decontamination,[20] the activity will diminish as the agents are depleted. On the other hand, coatings subject to weathering and abrasion would require biocidal additives that are well dispersed

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throughout the material, so that the biocidal properties are not lost upon damage of the surface.

Quaternary ammonium biocides are used in a variety of commercial applications ranging from cosmetic preservatives to hospital disinfectants and sanitizers.[21] These species have many advantages over other biocide classes (e.g. phenols and aldehydes) including: broad spectrum antimicrobial activity, effectiveness over a wide pH range, low human toxicity, low vapor pressure, amphiphilic solubility and lack of a detectable odor. Polymer surface modifiers (PSM) have recently been used to concentrate quaternary ammonium groups and other biocidal moieties at the surface-air interface,[22-24] driven primarily by low solubility and/or low surface energy.[25] One such case is a class of PSM comprised of copolymers containing fluoroalkyl groups adjacent to biocidal moieties.[23,24] The fluoropolymer segments were found to migrate through the bulk polyurethane and served to "chaperone" the chloroamide or quaternary ammonium biocidal components to the surface.

It was envisioned that homologous quaternary dimethylammonium compounds bearing hydrophobic *n*-alkyl groups and hydrophilic oxyethylene chains, both of variable length, would provide a series of novel biocides that allow adjustment in hydrophilicity and hydrophobicity as desired. Herein, we report a new class of discrete amphiphilic quaternary ammonium compounds that are highly effective antimicrobial additives in polyurethane films and do not exhibit any evidence for leaching biocidal agents. This work represents the first example of a biocidal additive with the ability to self-concentrate at the surface of a polymer film without exotic polymer functionalities. We further detail simple structural modifications that provide an unprecedented level of control over the additive's tendency to surface-concentrate, such that the properties of the coating can be fine-tuned to achieve maximum surface reactivity or to maintain biocidal activity upon damage or wearing.

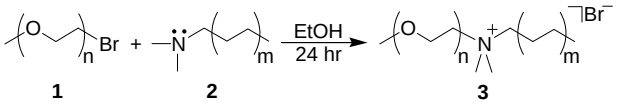

Figure 1. Synthetic scheme for the preparation of amphiphilic quaternary ammonium antimicrobials.

As depicted in figure 1, these amphiphilic biocides were prepared by reacting monodispersed methoxy-terminated oxyethylene bromides (**1**) with tertiary amines (**2**) in ethanol to afford the desired ammonium bromide compounds (**3**) in good yields. Minimum inhibitory studies were conducted and presented in Table 1 with contour plots depicting the data in a more visual format in figure 2.

Table 1. Minimum inhibitory concentration study results.

QAS MIC studies	Gram +	Gram -
$C_6QuatEO_1$	> 0.4 mg/mL	> 0.4 mg/mL
$C_6QuatEO_2$	> 0.4 mg/mL	> 0.4 mg/mL
$C_8QuatEO_1$	> 0.4 mg/mL	> 0.4 mg/mL
$C_8QuatEO_2$	> 0.4 mg/mL	> 0.4 mg/mL
$C_8QuatEO_3$	> 0.4 mg/mL	> 0.4 mg/mL
$C_{16}QuatEO_1$	0.0002 mg/mL	0.02 mg/mL
$C_{16}QuatEO_2$	0.0004 mg/mL	0.02 mg/mL
$C_{16}QuatEO_3$	0.001 mg/mL	0.1 mg/mL
$C_{16}QuatEO_4$	0.666667 mg/mL	0.4 mg/mL

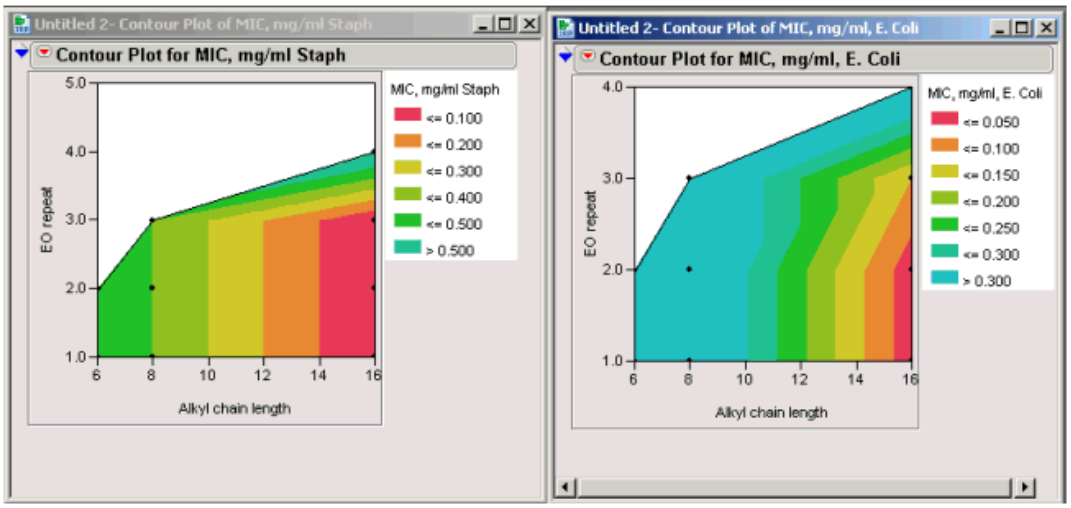

Figure 2. Contour plots of MIC results against two bacteria.

Table 2. Zone of inhibition data for quaternary ammonium bromides.

ZOI QAS	*Distance (mm)*
$C_6QuatEO_1$	2.4
$C_6QuatEO_2$	3.5
$C_6QuatEO_3$	0.3
$C_6QuatEO_4$	1.7
$C_8QuatEO_1$	2.4
$C_8QuatEO_2$	2.0
$C_8QuatEO_3$	2.3
$C_8QuatEO_4$	3.3
$C_{16}QuatEO_1$	1.2
$C_{16}QuatEO_2$	2.3
$C_{16}QuatEO_3$	1.4
$C_{16}QuatEO_4$	1.5

Table 3. Antimicrobial activity and XPS results for **3a-h**.

Entry	Product	m	n	Yield (%)	MIC[a] (mmol/L)		Log kill[b]		XPS[c]	
					S. aureus (G+)	*E. coli* (G-)	*S. aureus* (G+)	*E. coli* (G-)	%N obs.	%N calc
1	**3a**	2	1	83	9.3	9.3	5	3	0.1	0.05
2	**3b**	2	2	82	16.0	8.0	6	3	0.3	0.05
3	**3c**	2	3	96	14.0	7.0	3	1	0.1	0.05
4	**3d**	2	4	53	6.2	6.2	3	1	0.1	0.04
5	**3e**	3	1	79	0.7	2.0	7	6	0.5	0.05
6	**3f**	3	2	87	0.9	7.3	7	7	0.9	0.05
7	**3g**	3	3	94	1.3	6.5	5	4	0.3	0.04
8	**3h**	3	4	85	1.9	3.0	4	4	0.2	0.04

[a] Minimum inhibitory concentration. [b] Log reduction starting with 107 CFU/cm^2 on a coating of Hydrothane™ containing 1% biocide. [c] %N obs. is the weight % of biocidal nitrogen in the surface, excluding hydrogen, as observed by XPS. [d] %N calc. is the calculated number expected if the additive was evenly distributed throughout the coating, with no surface concentration.

All compounds were subjected to minimum inhibitory concentration (MIC) studies for effectiveness comparisons against the bacteria *S. aureus* (Gram-positive) and *E. coli* (Gram-negative), as shown in Table 1. Biocides possessing *n*-octyl alkyl groups (**3e-h**) were generally more effective antimicrobials than the corresponding analogs possessing n-hexyl groups (**3a-d**), particularly against *S. aureus*. Furthermore, the *n*-hexyl series was slightly more effective against *E. coli* than *S. aureus*, while the *n*-octyl series was significantly more effective against *S. aureus*. It is possible that the slightly longer and more hydrophilic *n*-octyl group is more effective than *n*-hexyl at disrupting the cell wall of Gram-positive bacteria, whereas the more complex Gram-negative cell wall is not as vulnerable to this functionality.

Variations in oxyethylene chain length were also found to affect activity. In the case of the *n*-octyl series, an increase in the oxyethylene chain length corresponded to a gradual decrease of antimicrobial activity against *S. aureus*. However, the compounds bearing oxyethylene chains of 1 or 4 repeat units (**3e** or **3h**) were significantly more effective against *E. coli* than those possessing chains of 2 or 3 units (**3f** or **3g**). In contrast, the *n*-hexyl derivatives bearing oxyethylene chains of 1 or 4 units showed increased activity against *S. aureus* (relative to those with chains of 2 or 3 units), while increasing the length of the oxyethylene chain corresponded to an increase in antimicrobial activity against *E. coli*. It was further noted that increasing the length of the oxyethylene chain assisted in solubilization, correlating to the predicted increase in hydrophilicity; however, it is likely that the amphiphilic nature of these molecules results in aggregation into micelles in aqueous solution. This could be hindering antimicrobial activity through obstruction of the biocidal functional groups, contributing to the apparent lack of congruity with respect to the observed trends in the effects of altering the oxyethylene chain length.

The effectiveness of **3a-h** as antimicrobial additives in coatings was conducted using Hydrothane™ polyurethane resin, selected for its hydrophilicity and lack of pigments, fillers, and any other additives that would hinder analysis. Films were cast at 1% (w/w) biocide loading and tested against the same bacteria employed in the MIC studies. Satisfactorily, all samples were found to retain their antimicrobial activity in the polyurethane, with log reduction as high as 7 (Table 3). Once again, the *n*-octyl derivatives were consistently more active than their n-hexyl counterparts, although,

contrary to the solution results, both series were found to be more effective against *S. aureus* than *E. coli* bacteria. Furthermore, while altering the length of the oxyethylene chain did not consistently affect the antimicrobial activity in solution, a clear trend emerges from the surface testing. In both the *n*-octyl and *n*-hexyl series, compounds bearing oxyethylene chains of 1 repeat unit are nearly (or equally) as effective as those with chains of 2 units, whereas a sharp decline in activity is displayed by compounds with chains of 3 or 4 units.

In order to shed light on this trend, x-ray photoelectron spectroscopy (XPS) analysis was conducted on each film (Table 3). A direct correlation between antimicrobial activity and biocide surface concentration was immediately evident. Figure 1 shows the effect of increasing oxyethylene chain length of the *n*-octyl series on log reduction of bacteria and observed surface concentration. The two most effective additives (**3e** and **3f**) were found to have the highest surface concentrations, with a 10- and 18-fold increase of the expected values, respectively. Furthermore, the trend in the effect of oxyethylene chain length on antimicrobial activity ($n = 2 \geq 1 >> 3 \geq 4$) is mirrored in the surface concentration data. From these observations, we have concluded that the long hydrophobic alkyl chains drive the surface concentration of the amphiphilic quaternary ammonium compounds via segregation from the hydrophilic polyurethane matrix. An increase in the length of the oxyethylene chain serves to decrease the hydrophobicity of the compound, which in turn lowers the energy gained by segregation and inhibits migration to the surface. That these effects are more pronounced in the *n*-octyl series provides additional evidence that the surface concentration is a consequence of the balance between the hydrophobicity of the *n*-alkyl group, the hydrophilicity of the oxyethylene chain, and, presumably, the hydrophilicity of the polymer matrix. Thus the molecule can be tailored to achieve a desired surface concentration in a given polymeric system by simply altering the lengths of the *n*-alkyl and oxyethylene groups, as shown in Figures 3 and 4.

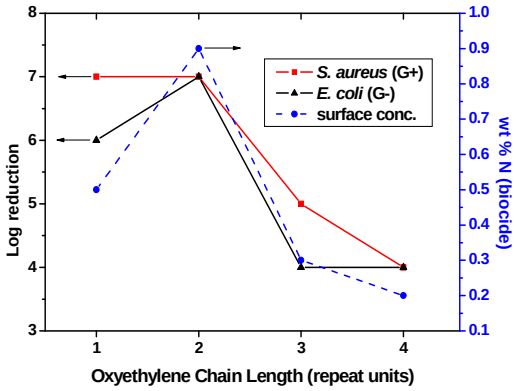

Figure 3. Effect of increasing oxyethylene chain length on Log reduction of *S. aureus*/*E. coli* and surface concentration of *n*-octyl bearing quaternary ammoniums.

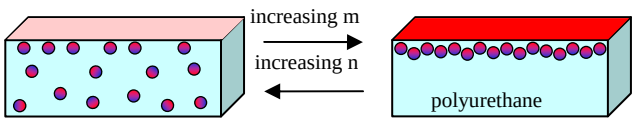

Figure 4. A graphic representation of the effect of altering the lengths of the *n*-alkyl (red) or oxyethylene (blue) groups on surface concentration.

The leaching of biocidal agent from a surface is a concern for any additive, particularly low molecular weight antimicrobials. Fortunately, films containing **3f** (chosen because of the high surface concentration) did not exhibit evidence of leaching when subjected to zone of inhibition studies (see Figure 5), tested both as prepared and after immersion in water for seven days. Also, samples did not show a decrease in antimicrobial activity, and the water was not found to inhibit bacterial growth nor contain **3f** within the limits of HPLC detection. This suggests that the additives are mobile and able to surface concentrate in the uncured polyurethane, but become "locked" in place once the resin has cured. Thus, diffusion of the antimicrobial agents out of the film is prevented despite the inherent water solubility.

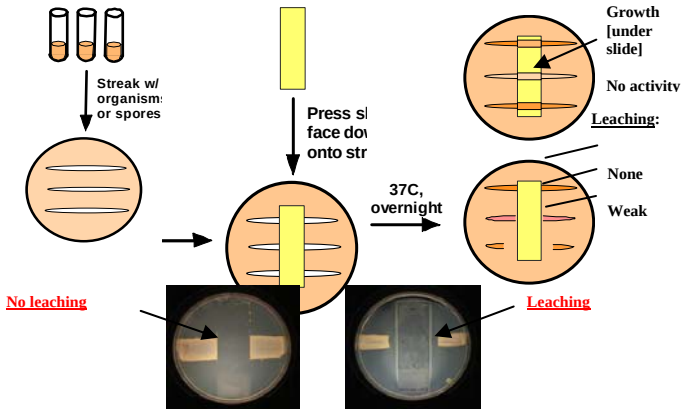

Figure 5. Zone of inhibition evaluation: permanence in model paint.

In conclusion, a novel class of quaternary ammonium compounds was designed and synthesized that self segregate to the polymer-air interface and was proven to be effective antimicrobials against both Gram-positive and Gram-negative bacteria. To our knowledge, this is the first report of non-polymeric biocidal additives capable of self-concentrating at the surface of a polymeric coating. Altering the lengths of the alkyl groups and oxyethylene chains provided a unique method of controlling the surface concentration of these molecules within polyurethane films, as demonstrated by XPS analysis, affording an unprecedented ability to "dial in" antibacterial properties to a polymer surface as desired for a specific application. Methods of refining control over surface concentration and improving antimicrobial activity through further alterations of the n-alkyl groups and other functionalities, as well as the extension of this work into other resin systems, are currently under investigation.

Preparation and Evaluation of Non-Ionic Amphiphilic Phenolic Biocides in Urethane Hydrogels

Phenolic compounds are common in naturally occurring biologically active agents and often represent a large fraction of the extracts obtained from natural products.[26] These compounds are now finding application as antimicrobial additives to coatings and films for a variety of markets, such as the food industry. A specific example includes a clever introduction of essential oil extracts of oregano to an alginate-based film applied to beef muscle slices for enhanced preservation.[27] In addition to their broad application as biocides and as synthetic intermediates in the preparation of pesticides, phenolic-based materials are also added liberally to coatings as antioxidants, such as the monomer (i.e. 2,4-ditert-butylphenol, 2,4-DTBP) and oligomeric compound (i.e. nonylphenol disulfide, ETHANOX 323^{TM}) which are available commercially.[28] Recent studies by Boudjouk[29] and Yoon[30] report the use of phenolic biocides in the preparation of active antimicrobial coatings. Specifically, the Thomas group incorporated Triclosan® (5-chloro-2-(2,4-dichlorophenoxy) phenol) as a functional pendant group to the silicone backbone for use in the preparation of new antifouling coatings. Yoon's group polymerized vinyl monomers having phenol pendant groups to produce antimicrobial polymers.

As a matrix material, the versatile, inexpensive, and readily available polyurethane has found wide use in the preparation of antimicrobial films and coatings, and several excellent recent studies exist. A polyether-type polyurethane film containing well-dispersed silver nanoparticles was prepared by Hsu[31] and found to impart increased biostability when implanted in a rat subcutaneous model. Similarly, Piozzi[32] has developed a dual-antimicrobial agent polymer system comprised of a new silver-complexing polyurethane and ciprofloxacin antibiotic additive for coating medical devices. Most recently, the Piozzi group[33] report the preparation of antibiotic-releasing polyurethane coatings for central venous catheters, which may ultimately prevent bacteria colonization and the emergence of bacterial resistance. In an elegant application of surface active biocides, block urethane polymers were synthesized by the Wynne group containing pendant hydantoin groups that are readily converted to the active biocidal compound when treated with hypochlorite.[34]

Our interest lies in the synthesis, characterization and evaluation of custom antimicrobial compounds designed for both high biocidal activity at the surface against a variety of pathogens and compatibility with commercial carrier liquids and common polymer resins.[1] In this current work, a polyurethane hydrogel was chosen as the polymer matrix due to its exceptional versatility and unique water absorption characteristics, and the specific urethane hydrogel employed is capable of absorbing from 5 to 25% water by weight. The phenolic biocidal compounds possess polar polyether segments to promote water-solubility and resin compatibility along with non-polar alkyl chains to promote orientation and present the phenolic OH at the air-surface interface. Coatings having these unique characteristics are suitable for eventual use in food service and storage areas and in hazardous waste containment and temporary storage vessels to deter bacterial growth.

Within we report the synthesis and evaluation of a novel series of amphiphilic phenolic biocides. Each biocide was first evaluated for the minimum inhibitory concentration (MIC) employing a standard aqueous solution test protocol.[35] Each of the biocides was then blended into a hydrophilic urethane hydrogel in THF, and coatings were solvent cast and analyzed for antimicrobial activity. This is the first report of a series of amphiphilic phenolic biocides blended within a urethane hydrogel. Biocidal activity results from film studies were obtained using a new film testing protocol and subsequently compared to solution MIC values.

Because of their broad spectrum of antimicrobial activity, benign environmental impact and current commercial utility, a non-ionic amphiphilic phenol biocidal moiety was selected for this study. The amphiphilic biocides were synthesized by condensation of 4-hexylresorcinol with the corresponding hydroxyl-terminated monomethyl-PEG in the presence of a catalytic amount of acid in refluxing toluene (Figure 6). Although this reaction is predicted to afford a mixture of products, the desired product **6** was obtained as the major product along with the di-substituted and alternate 3-substituted product in greatly dimished quantites. It is believed that the steric effects of the *n*-hexyl substituent of the resorcinol, selectively directs condensation to the one position resulting in the formation of **6** almost quantitatively. The by-products, acid catalyst and unreacted starting materials are removed by flash column chromatography, resulting in isolation of the desired product in significant purity.

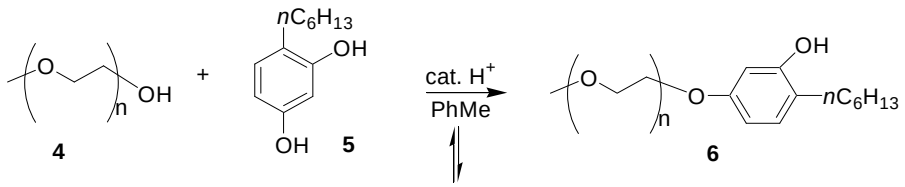

Figure 6. Synthetic scheme for amphiphilic phenol biocide series.

General procedure for preparation of (**6**): In a 25-mL, round-bottomed flask equipped with magnetic stir bar, Dean-Stark trap and condenser were placed 4-Hexyl-benzene-1,3-diol (4-hexylresorcinol) (0.59 g, 6.25 mmol), an ethyleneoxide monomethylether (6.25 mmol), *p*-toluenesulfonic acid (0.01g, 0.008 mmol), and 20 mL of toluene. An additional 7 mL of toluene was placed in the Dean-Stark trap to prevent taking the pot volume too low. The solution was allowed to reflux vigorously for 24 hours in an oil bath. The resulting solution was allowed to cool to room temperature and concentrated using the rotary evaporator. The resulting oil was eluted through a silica gel column employing a Hexane/EtOAc (1:1) solvent system, and the desired product eluted in the first fraction.

2-Hexyl-5-(2-methoxy-ethoxy)-phenol (**6a**): FTIR: 3362, 2950, 2930, 2858, 1606, 1519, 1463, 1376, 1297, 1221, 1162, 1114, 1055, 972 cm^{-1}. ^{1}H NMR ($CDCl_3$): 6.91 (d, *J*=9, 1H), 6.34 (d, *J*=2, 1H), 5.76 (d, *J*=5, 2H), 3.54 (d, *J*=5, 2H), 3.37 (s, 3H), 2.48 (t, *J*=8, 2H), 1.55-1.50 (m, 2H), 1.34-1.25 (m, 6H), 0.87 δ (t, *J*=7, 3H). ^{13}C NMR ($CDCl_3$): 154.3, 154.2, 130.7, 121.3, 107.5, 102.9, 73.4, 61.5, 58.7, 31.7, 29.2, 29.1, 22.6, 14.1 δ. Anal. Calcd for $C_{15}H_{24}O_3$: C, 71.39; H, 9.59. Found: C, 71.68; H, 9.31.

2-Hexyl-5-(2-(2-methoxy-ethoxy)-ethoxy)-phenol (**6b**): FTIR: 3346, 2961, 2922, 2858, 1622, 1519, 1459, 1376, 1301, 1225, 1166, 1118, 968 cm^{-1}. ^{1}H NMR ($CDCl_3$): 6.92 (d, *J*=9, 1H), 6.36 (d, *J*=2, 1H), 6.32 (d, *J*=6, 1H), 5.37 (bs, 1OH), 3.78 (t, *J*=6, 2H), 3.66 (t, *J*=5, 2H), 3.63-3.59 (m, 4H), 3.42 (s, 3H), 2.50 (t, *J*=6, 2H), 1.55-1.51 δ (m, 2H). ^{13}C NMR ($CDCl_3$): 154.6, 154.4, 130.6, 120.9, 107.4, 102.8, 72.2, 71.9, 69.9, 61.8, 58.9, 31.7, 30.0, 29.2, 29.1, 22.6, 14.1 δ. Anal. Calcd for $C_{17}H_{28}O_4$: C, 68.89; H, 9.52. Found: C, 68.73; H, 9.44.

2-Hexyl-5-(2-(2-(2-methoxy-ethoxy)-ethoxy)-ethoxy)-phenol (**6c**): FTIR: 3345, 2950, 2913, 2851, 1626, 1601, 1519, 1459, 1380, 1348, 1301, 1217, 976 cm^{-1}. ^{1}H NMR ($CDCl_3$): 6.91 (d, *J*=9, 1H), 6.41 (d, *J*=2, 1H), 6.33 (d, *J*=6, 1H), 3.77-3.73 (m, 2H), 3.70-3.56 (m, 10H), 3.38 (s, 3H), 2.50 (t, *J*=6, 2H), 1.55-1.49 (m, 2H), 1.33-1.27 (m, 6H), 0.87 δ (t, *J*=6, 3H). ^{13}C NMR ($CDCl_3$): 155.0, 154.7, 130.5, 120.7, 106.9, 102.6, 72.4, 71.7, 70.5, 70.3, 70.2, 61.6, 58.9, 31.8, 30.0, 29.3, 29.2, 22.6, 14.1 δ. Anal. Calcd for $C_{19}H_{32}O_5$: C, 67.03; H, 9.47. Found: C, 66.85; H, 9.27.

2-Hexyl-5-(2-(2-(2-(2-methoxy-ethoxy)-ethoxy)-ethoxy)-ethoxy)-phenol (**6d**): FTIR: 3342, 2930, 2858, 1618, 1606, 1523, 1459, 1344, 1301, 1253, 1198, 1094, 980 cm^{-1}. ^{1}H NMR ($CDCl_3$): 6.91 (d, *J*=9, 1H), 6.41 (d, *J*=2, 1H), 6.33 (d, *J*=6, 1H), 3.75 (t, *J*=6, 2H), 3.68-3.55 (m, 14H), 3.37 (s, 3H), 2.50 (t, *J*=9, 2H), 1.55-1.52 (m, 2H), 1.33-1.24 (m, 6H), 0.87 δ (t, *J*=6, 3H). ^{13}C NMR ($CDCl_3$): 155.0, 154.7, 130.5, 120.7, 106.9, 102.7, 72.4, 71.8, 70.5 (overlapping peak), 70.4, 70.3, 70.1, 61.7, 58.8, 31.8, 30.1, 29.3, 29.2, 22.6, 14.1 δ. Anal. Calcd for $C_{21}H_{36}O_6$: C, 65.60; H, 9.44. Found: C, 65.86; H, 9.38.

2-Hexyl-5-(polyethyleneglycol monomethyl ether)-phenol (**6e**): (PEG MW~750) FTIR: 3347, 2957, 2926, 2851, 1620, 1604, 1452, 1345, 1315, 1259, 987 cm^{-1}. ^{1}H NMR ($CDCl_3$): 6.84 (s, 1H), 6.28 (d, *J*=6, 1H), 6.19 (d, *J*=6, 1H), 4.10 (t, *J*=4, 2H), 3.76-3.50 (m, 64H), 2.55 (t, *J*=6, 2H), 1.64-1.60 (m, 2H), 1.33-1.29 (m, 6H), 0.96 δ (t, *J*=6, 3H). ^{13}C NMR ($CDCl_3$): 157.6, 157.3, 130.2, 118.2, 106.5, 101.1, 73.1, 73.0, 70.7 (overlapping peaks), 70.6 (overlapping peaks), 70.5 (overlapping peaks), 53.9, 32.7, 32.5, 32.0, 29.5, 25.5, 25.4, 13.9 δ.

All newly prepared amphiphilic phenolic biocides were subjected to minimum inhibitory concentration (MIC) evaluations against both Gram-positive and Gram–negative bacteria (Table 4). Further MIC evaluations were undertaken to determine the effectiveness of prepared phenolic biocides (**6a**-**e**) against a broader spectrum of potential pathogens (Table 5). MIC testing procedure was conducted by inoculating 10^7 cfu of mid-log phase growth (18h at 37˚C) bacteria of the indicated strain into fresh culture tubes containing sterile LB media, followed by addition of varying concentrations of the biocide to be evaluated. Cultures were then incubated for 18h at 37˚C, and evaluated for the presence of bacterial growth as indicated by turbidity within the media.

Table 4. Initial minimum inhibitory concentration studies for Phenols.

Phenol	***S. aureus***	***E. coli***
C_6PhenolEO$_1$	0.18 mg/mL	0.31 mg/mL
C_6PhenolEO$_2$	0.31	0.31
C_6PhenolEO$_3$	0.63	2
C_6PhenolEO$_4$	0.63	2.5

Table 5. Minimum inhibitory concentration* (μg/mL) of aqueous phenolic biocides.

Entry	***1***	*Product*	*Yield (%)*	*S. aureus (Gram +)*	*B. anthracis (Sterne)(Gram +)*	*E. coli (Gram -)*	*S. typhimurium (Gram -)*
1	*n*=1	**6a**	73	124	170	52	173
2	*n*=2	**6b**	78	113	165	67	186
3	*n*=3	**6c**	64	75	167	47	143
4	*n*=4	**6d**	55	83	189	81	132
5	*n*=16	**6e**	48	102	174	94	129

*mg of biocide required to neutralize 1 mL of respective bacteria at 10^5 CFU/mL

Although, in general, all were effective in the lysing of bacteria, none of the phenols (**6a-e**) were outstanding performers when directly compared to published MIC data for commercial phenolic biocides.[37] However, recent findings in our laboratory suggest that one must be cautious in dismissing the utility of an apparent high-MIC biocide, as there is often a poor correlation of solution antimicrobial activity and the effectiveness of the same biocide in a film/coating.[37] All phenols are inactivated by inclusion in micelles, which can occur in solutions when the detergent/surfactant (non-ionic and anionic) concentration exceeds the critical micellization concentration.[26] We believe that the elevated MIC data reflects the presence of solution micellular formation in competition with antimicrobial activity performance. However, it should be noted that the MIC data of the new biocides reflected the significantly enhanced activity against E. coli, the Gram-negative bacterium, over the Gram-positive bacteria examined. Phenolic biocidal compounds are reported to be membrane-active agents,[30,38] ultimately rupturing the cell membrane and releasing the intracellular constituents.

Phenolic biocides **6a-e** used in the MIC study were also incorporated into a hydrophilic polyurethane and evaluated as an antimicrobial film additive against the same bacteria used in solution testing. The choice and compatibility of the polymer matrix is critical to the success of the resulting active coating, because antimicrobial activity of compounds in polymers has been shown to depend on molecular diffusion of the antimicrobial agents in the matrix.[39] Selection of the urethane was based on the desire to have a one-component hydrophilic urethane with relatively low glass transition temperature (T_g). Glassy polymers are considered inferior matrix materials. In addition, phenolic biocidal compounds are incompatible with the reactive isocyanates that are inherently present in the two-component system.

Films were prepared by combining 0.80 g Hydrothane^{TM} with 25 mL of freshly distilled THF and stirring for 4 hours, at which time the polymer completely dissolved. To the dissolved Hydrothane^{TM}, a solution consisting of 0.008 g (**6**) dissolved in 1 mL of di-H_2O

was added dropwise, resulting in a final loading of ~1 wt% (w/w) with respect to polymer solids. The final solution was allowed to stir for an additional 30 min, and films were solvent cast by adding 1 mL solution via pipette to a pre-cleaned microscope glass slide. The glass slide was held overnight in a sterilized covered Petri dish to slow the rate of evaporation. The resulting films were rinsed with 5 mL di-H_2O to clean the surface of any un-included ammonium salt prior to subsequent examination and antimicrobial testing.

To address the permanence of phenolic compounds within the prepared films, zone of inhibition (ZOI) tests were conducted (Table 6). Tests were conducted by placing prepared films containing biocide upside down on LB agar plates upon which a lawn of bacteria had been prepared. Plates were incubated for 18h at 37˚C, and zones of clearing surrounding the films were measured (Figure 7). ZOI tests indicated that ethylene oxide chain length was the major factor determining leaching of biocide from prepared films, with a chain length of n=3 exhibiting the smalled ZOI, and thus greatest permanence within the tested films.

Table 6. Zone of inhibition data for phenols.

ZOI Phenols	***Compound ID***	***Distance (mm)***
C_6PhenolEO$_1$	**6a**	12.8
C_6PhenolEO$_2$	**6b**	15.7
C_6PhenolEO$_3$	**6c**	6.1
C_6PhenolEO$_4$	**6d**	12.1

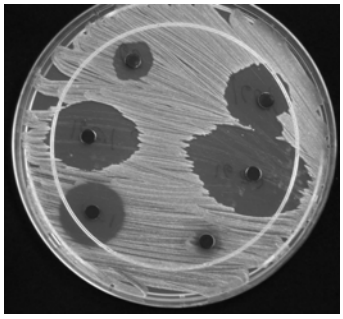

Figure 7. Image of a zone of inhabitation study plate.

The method for the evaluation of each coating's antimicrobial activity is a well established serial dilution screening that has been employed previously. [40,41] Briefly: to a 1 L Erlenmeyer flask equipped with a stir bar was added 25.7 g Letheen broth™ (Difco Laboratories, Detroit, MI) and 1 L Milli-Q® filtered water. The mixture was stirred over low heat for 30 minutes. Aliquots (4.5 mL) of the resulting solution were added to autoclavable culture tubes (~200) to be used in subsequent serial dilutions. The test tubes were covered with plastic lids and autoclaved at 121 °C (and 15 psi) for 25 minutes. Letheen broth was selected for its ability to neutralize the biocidal effect of phenols with sorbitan monooleate, so that continued antibacterial action would not occur after the serial dilution step. Preparation of bacteria: *Staphylococcus aureus* (ATCC 12598), *Escherichia coli* (ATCC 0157:H7), *Salmonella typhimurium* (ATCC 14028) and *Bacillus*

anthracis (ATCC 34F2) cells were each grown in our laboratory according to standard microbiological techniques. Bacteria were harvested from an agar plate by removing a single CFU (colony forming unit) with a sterile inoculating loop, and placing it in Letheen broth. The culture was incubated at 28–30 °C overnight in a shaking incubator. The cells were then pelleted by centrifugation at 3000 RPM and 18 °C. The cells were then re-suspended in 0.5% saline solution to achieve a density of about 10^9 CFU/mL as determined by McFarland turbidity standards. A 1 μL aliquot was taken from a solution of each bacteria (concentration 10^9) and applied directly to the coating on a microscope slide, resulting in the delivery of 10^7 CFU/cm^2. The slides were placed in sterile Petri dishes with a piece of hydrated filter paper in the bottom of each dish. The use of the hydrated filter paper prevents the death of the bacteria by desiccation. After allowing the slides to incubate for two hours, the coating surface was thoroughly swabbed using two sterile swabs and vortexed in a 4.5 mL solution of previously sterilized Letheen broth. Letheen broth was selected because it contains sorbitan monooleate, which neutralizes the antimicrobial activity of any phenol that may have been extracted by aggressive swabbing, thus preventing additional kill once recovered. The initial tube is then serially diluted by extracting 0.5 mL and depositing it into the subsequent tube for a total of seven tubes. The tubes were allowed to incubate at 35 °C for 24 hrs before they were read. Positive growth was indicated by the presence of string-like filamentous growth of colonies of bacteria in solution, not mere murkiness, which may result from other forms of contamination. All data reported are an average of triplicates, and data is reported as log-reduction from a starting concentration of 10^7 CFU/mL.

Table 7. Biological activity results* of phenolic biocides in polyurethane hydrogels.

Film containing biocide	*Loading (w/w)%*	*E. coli (Gram -)*	*S. aureus (Gram +)*	*S. typhimurium (Gram +)*	*B. anthracis (Sterne) (Gram +)*
6a	0.25	4	3	3	2
6b	0.25	4	4	2	2
6c	0.25	4	4	3	2
6d	0.25	3	3	2	2
6e	0.25	3	4	2	3
6a	0.5	6	7	5	4
6b	0.5	5	5	5	3
6c	0.5	5	6	4	4
6d	0.5	5	5	4	4
6e	0.5	5	6	3	5
6a	1.0	6	7	5	3
6b	1.0	6	5	4	3
6c	1.0	5	6	4	4
6d	1.0	4	5	3	3
6e	1.0	5	6	3	5
6a	2.0	5	6	5	3
6b	2.0	6	5	3	2
6c	2.0	4	4	4	3
6d	2.0	4	5	3	2
6e	2.0	5	5	2	4

*Values 1-7 describe the log-reduction from a starting concentration of 10^7 CFU/cm^2

The biological activity of our custom phenols when incorporated into urethane hydrogels is summarized in Table 7, where the numbers 1-6 represent the log kill reductions in

colony-forming units when starting with a 10^7 concentration. For example, a log 4 kill represents a reduction of 99.99% in bacteria capable of forming colonies. Because there are currently no standard antimicrobial coating test methods, our antimicrobial activities were evaluated utilizing a new method developed in our laboratory. Standard test methods exist to evaluate fabrics against a variety of bacteria at concentrations of $10^5/cm^2$; however, the different scope of our application has required modification of this original procedure. In this current work, we wished to demonstrate antimicrobial activity that one would likely encounter in a biological attack and have therefore increased the bacteria loading to ~10^7 CFU/cm^2. The coatings were inoculated, allowed to incubate for two hours, and evaluated by swabbing followed by serial dilution and incubation.

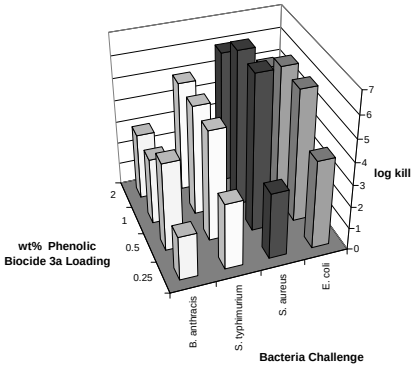

Figure 8. Antimicrobial action versus challenge for amphiphilic phenolic biocide **6a** in polyurethane hydrogel.

Upon analysis of the data shown in Table 7, it was concluded that compound **6a** exhibited best overall antimicrobial action (Figure 8). Although for *B. anthracis*, the performance of **6e** was greater. It was concluded that the increase in ethylene oxide length had a more dramatic effect on the antimicrobials when subjected to *B. anthracis* over other bacteria examined. For all biocides examined, there appeared to be a slight increase in activity when the phenolic biocide concentration was increased from 0.25% (w/w) to 0.5 and 1%; however, increasing the phenolic biocide concentration above 1 wt% results in very little increase in activity. This plateau of biocidal activity at higher agent concentrations is not entirely understood. A recent report,[36] speculates that the reduced kill at higher concentrations results from the formation of micelles, which places limits on solution biocidal activity or in this case aggregate and concentrate at the urethane-air interface thus inhibiting coating surface activity.

In the challenge with the Gram-negative bacteria, *E. coli*, optimum results were obtained in films containing 0.5 and 1% loading of amphiphilic phenolic biocide. Diminished activity resulted from both higher and lower wt% loadings. Compound **6a** shows superior overall activity among the group of biocidal compounds tested. Three common pathogenic Gram-positive bacteria were also evaluated, including the spore former *B. anthracis* (Sterne). The amphiphilic phenol biocides were more active against both *S. aureus* and *S. typhimurium*, than *B. anthracis*. This is to be expected, because phenols are known to be less active toward spore-forming bacteria. Although the results from exposure of films to *S. typhimurium* were slightly dimished when compared to that of *S.*

aureus, trends were similar and the effects of loading were comparable for all samples examined.

Structural features of the amphiphilic phenolic biocides, such as the length of the tethered PEG chain, were found to significantly affect the antimicrobial activity of the resulting biocide. Biocidal testing results indicate that a lengthening of the PEG chain results in a significantly diminished antimicrobial activity. This reduced activity was attributed to agent mobility and solubility: specifically, the ability of the phenol to remain mobile within the curing resin and present its active phenolic OH subunit at the surface. The more hydrophilic longer PEG chain, as observed in compound **6e**, is believed to remain deeper in the bulk of the coating rather than self-stratifying to the air-coating interface, as was desired for making surface-active biocidal coatings. The Yoon group observed a similar effect in a biocidal system, where the relative biocidal activity among a series of structurally similar agents was attributed at least in part to the compound's hydrophilic nature and ease of diffusity in media.[30] The hydrophobic hexyl alkyl chain substituent is believed to assist in the mobilization of the biocidal moiety to the coating/air interface, thus resulting in the increased bioactivity. Less hydrophilic tethers, as observed in phenols **6a-d**, provide more flexibility and consequently greater freedom for movement within the coating upon application and curing. This increased flexibility allows for maximum orientation of the active functional group with respect to surface-residing contamination/bacteria and thus may actually promote self-concentration at the surface ultimately resulting in a more viable and effective antimicrobial coating.

A series of novel non-ionic amphiphilic phenolic molecules were prepared and evaluated for their anti-microbial activity in solution using a standard technique, which reports biocidal activity as MIC. The functional phenols were also evaluated for surface anti-microbial activity in polymer blends with a urethane hydrogel, using a new testing protocol developed in our lab. Although solution MIC were found to be unremarkable for the phenolic series, high surface anti-microbial activity could be obtained and is expressed as a log kill reduction in colony forming units starting from an initial bacterial concentration of 10^7 CFU. The structural features of the phenolic biocide were found to contribute significantly to the observed anti-microbial activity. Moreover, highest activity was observed in samples containing the phenolic compound with shortest ethylene oxide polar structural feature and, therefore, the highest mobility in the polar urethane resin. Coatings having these characteristics are potentially useful in the food storage and medical industries.

Preparation and Evaluation of Alkyl-Pyridinium Biocides

Pyridiniums are well utilized in a variety of commercial health products such as mouth rinse and toothpaste. They have also demonstrated broad spectrum antimicrobial activity, yet pose very few environmental and health concerns. The synthesis of a variety of substituted pyridiniums were conducted as is depicted in Figure 9. Upon condensation of 4-hydroxypyridine with the respective bromo-monomethyloxyethylene ether the intermediate pyridine resulted. Subsequent treatment with an alkyl halide such as

bromohexane affords the desired amphiphilic pyridium. The oxyethylene was varied where n = 1,2,3,4,17. This series provides an analogous comparison between the phenol and pyridinium series. One advantage alkyl-pyridniums have over phenols is that they offer a more general compatibility with a variety of resin systems, which would not be compatible with -OH sensitive systems. Likewise, the length of the alkyl bromide can also be varied if desired to alter the hydrophobic component of the product.

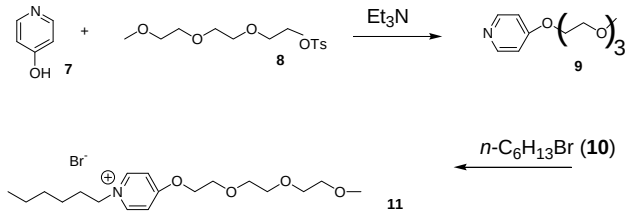

Figure 9. Synthetic scheme for alkyl-pyridinums.

Bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA.) *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 11229) were used for bacterial Gram-positive and Gram-negative challenges, respectively. Luria-Bertani (LB) media (Difco Laboratories, Detroit, MI), prepared as per the manufacturer's specifications, was used as a bacterial growth and dilution medium for preparation of bacteria for Gram-positive and Gram-negative challenges.

To determine the minimum inhibitory concentration (MIC) of the perspective biocide, compounds were weighed and dissolved in sterile water. Each compound was then added to Luria-Bertani (LB) media at varying concentrations. Bacteria were grown at 37 ˚C. Log phase cells were harvested by centrifugation, counted on a hemocytometer using bright field microscopy, pelleted by centrifugation at 4000 x g for 10 min, and resuspended in a 0.5 % NaCl solution at a concentration of 1 x 10^7 cfu/mL. To the mixture of LB and biocide was added a 10 μL aliquot containing 1 x 10^5 colony forming units (CFUs) of either *Staphylococcus aureus* (ATCC 25923) for Gram-positive or *Escherichia coli* (ATCC 11105) for Gram-negative. Cultures were then incubated for 18 hr at 37 ˚C with agitation and examined for turbidity. MIC was determined to be the lowest concentration of biocide that prevented visible bacterial growth at 18 hr.

Additionally, zone of inhibition (ZOI) testing was conducted to determine the stability of the additives within the coating. Coatings were prepared containing 1% by weight additive. Disks were punched out and placed coating side down on agar plates upon which a lawn of bacteria had been prepared. Plates were incubated for 18 hr at 37 ˚C, and zones of clearing around the disks were measured to determine the extent of leaching of the additives.

To determine activity of prepared coatings against viral pathogens, viral pathogen homologues were exposed to coatings, and log kill was determined. Pseudorabies virus (ATCC VR-135) was used as a enveloped virus analog, and Feline calicivirus (ATCC VR-529) was used to simulate non-enveloped viral pathogens. Activity against viral pathogens was tested as follows. Permissive cell lines are grown according to ATCC

guidelines in 75 cm^2 flasks. At confluency, media is removed from cells and replaced with DMEM lacking serum, and virus is added. Virus is allowed to attach to cells for 1 hour at room temperature, followed by addition of DMEM +5% FBS. Cells are placed at 37˚C with 5% CO_2 and allowed to grow until approximately 75% of cell exhibit viral induced cytopathic effects (CPE). Virus is harvested via 3 sequential freeze-thaw cycles, and frozen at -80˚C in 1 mL aliquots. Viral load is determined via traditional plaque assay. For surface testing, approximately 10^7 pfu of virus are added to the surface of interest and incubated at room temperature for two hours. Surfaces are then washed thoroughly using DMEM containing no serum. Eluant from the wash is then added to permissive cells that have been grown to confluency in 24 well plates, followed by serial dilution and 48h incubation at 37˚C with 5% CO_2. Log kill is determined by counting plaques in a traditional plaque assay.

MIC tests showed a range of results as can be seen in Table 8 and Figure 10. A clear patterned emerged regarding rational design of antimicrobial pyridinium compounds. Both ethylene oxide (EO) and alkyl chain lengths had an effect on antimicrobial activity. Too short or too long EO chains greatly reduced the antimicrobial activity, with an EO repeat chain length of two being ideal for both Gram-positive and Gram-negative tests. Alkyl chains showed a wider range of effective lengths, with a general trend toward longer chains having a greater antimicrobial effect.

Table 8. MIC results in mg/mL for pyridinium coumpous against *S. aureus* (G+) and *E. coli* (G-).

Pyridinium	***Compound ID***	***S. aureus***	***E. coli***
Pyridinium C_{16}	**12**	0.012	3.9
Pyridinium C_{12}	**13**	15.6	62.5
Pyridinium C_6	**14**	0.122	3.9
C_6PyridiniumEO$_1$	**15**	2.5	5
C_8PyridiniumEO$_1$	**16**	>5	>5
C_{16}PyridiniumEO$_1$	**17**	0.18	2.5
C_6PyridiniumEO$_2$	**18**	2.5	1.25
C_8PyridiniumEO$_2$	**19**	0.1	0.625
C_{16}PyridiniumEO$_2$	**20**	0.31	<0.31
C_6PyridiniumEO$_3$	**11**	2.5	2.5
C_8PyridiniumEO$_3$	**21**	2.5	>5
C_{16}PyridiniumEO$_3$	**22**	0.625	2.5
C_6PyridiniumEO$_4$	**23**	2.5	5
C_8PyridiniumEO$_4$	**24**	5	>5
C_{16}PyridiniumEO$_4$	**25**	>5	2.5

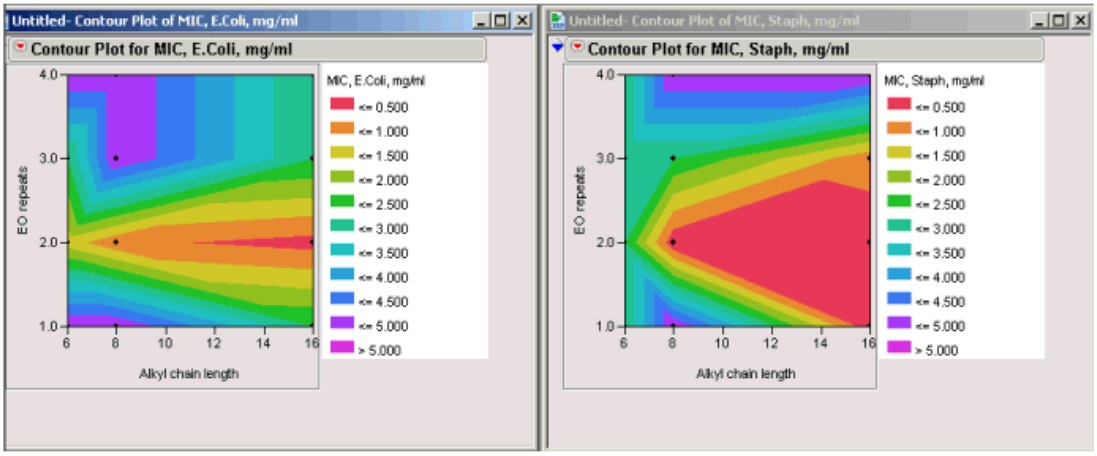

Figure 10. Contour plots of effective biocidal moieties against bacterial challenges.

Results for the stability of the additives within the coating are seen in Table 9. ZOI data indicates a small amount of leaching of pyridinium compounds occurs. Distinct conclusions regarding relative leaching rates between different compounds are difficult to make due to the large difference in antimicrobial activity among the compounds. The data shows that compounds with greater antimicrobial activity, as evidenced by lower MIC values, had larger zones of clearing. However, this does not indicate a greater degree of leaching only that leaching has occurred.

Table 9. ZOI testing of pyridinium compounds against *S. aureus*.

ZOI Pyridiums	***Compound ID***	***Distance (mm)***
C_6PyridiniumEO$_1$	**15**	0
C_6PyridiniumEO$_2$	**18**	0
C_6PyridiniumEO$_3$	**11**	3
C_6PyridiniumEO$_4$	**23**	1.7
C_8PyridiniumEO$_1$	**16**	2.4
C_8PyridiniumEO$_2$	**19**	2.9
C_8PyridiniumEO$_3$	**22**	2.3
C_8PyridiniumEO$_4$	**24**	3.3
C_{16}PyridiniumEO$_1$	**17**	3.2
C_{16}PyridiniumEO$_2$	**20**	2.4
C_{16}PyridiniumEO$_3$	**23**	3.5
C_{16}PyridiniumEO$_4$	**25**	0

Viral surface testing results indicate that coatings are effective against Pseudorabies virus (Figure 11), but showed no activity against Feline calicivirus (not shown). This result is consistent with previous reports that quaternary ammonium compounds are effective in deactivating enveloped viruses, but are totally ineffective against non-enveloped viruses. This is consistent with the mode of action employed by compounds such as quaternary

ammoniums. They disrupt phospholipid membranes leading to ion leakage. This ion leakage leads to the inability for viruses to infect new cells and to cell death for bacteria. Enveloped viruses are composed of an outer membrane of phospholipids acquired from the host cell, while non-enveloped viruses lack such a membrane.

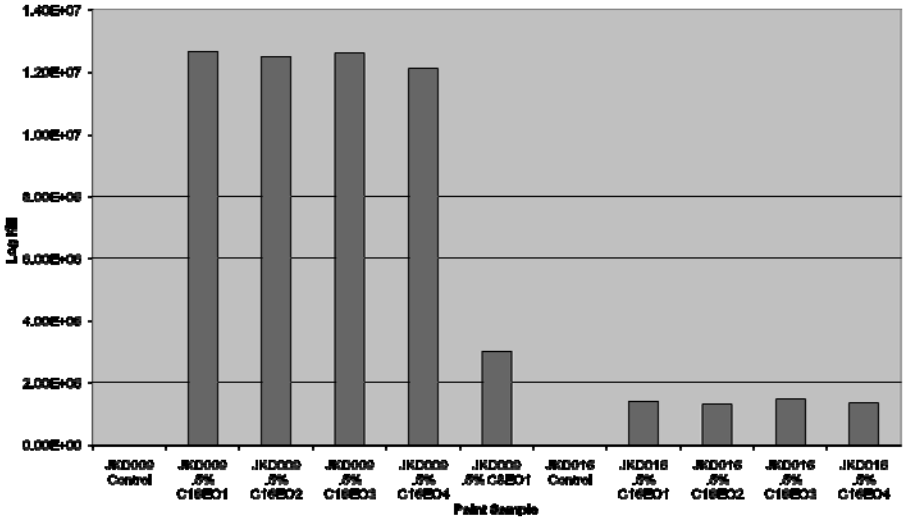

Figure 11. Results of surface viral testing against Pseudorabies virus.

A series of novel alkyl-pyridinium biocides were prepared and evaluated for their anti-microbial activity. The biocides were in solution using a standard MIC technique, evaluated for permanence in coatings, and evaluated for effectiveness against viral pathogen homologs. As with phenolic biocides, structural features of the alkyl-pyridinium biocides were found to contribute significantly to the observed anti-microbial activity. Highest activity was observed in samples containing the alkyl-pyridinium compound with longer alkyl chain lengths ($C_{14\text{-}16}$) and ethylene oxide polar chain length of 2-3 repeat units. ZOI studies indicate that alkyl-pyridinium biocides leach from prepared coatings to various degrees, although a clear pattern relating structure of biocides to degree of leaching was not observed. In addition, alkyl-pyridinium biocides in prepared coatings showed clear ability to reduce the amount of active enveloped virus (PRV) by 6-7 logs, but, as expected, showed to activity against virus lacking a phospholipid membrane.

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